

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS

Event-specific Method for the Quantification of Maize DAS-40278-9 by Real-time PCR

Validation Report and Validated Method

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EUROPEAN COMMISSION
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Event-specific Method for the Quantification of Maize DAS-40278-9 by Real-time PCR

Validation Report

7 November 2012

European Union Reference Laboratory for GM Food and Feed

Executive Summary

In line with its mandate¹ the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting and quantifying maize event DAS-40278-9 (unique identifier DAS-40278-9). The validation study was conducted according to the EU-RL GMFF validation procedure [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>] and the internationally accepted guidelines².

In accordance with current EU legislation¹, Dow AgroSciences LLC has provided the detection method and the positive and negative control samples (genomic DNA from maize seeds harbouring the DAS-40278-9 event as positive control DNA, genomic DNA from conventional maize seeds as negative control DNA). The EU-RL GMFF prepared the validation samples (calibration samples and blind samples at different GM percentage [DNA/DNA]), organised an international collaborative study and analysed the results.

The study confirms that the method meets the method performance requirements as established by the EU-RL GMFF and the ENGL in Annex I-2.C.2 to Regulation (EC) No 641/2004¹ and it fulfils the analytical requirements of Regulation (EU) No 619/2011.

This report is published at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

¹ Regulation (EC) No 1829/2003 of 22 September 2003 "on genetically modified food and feed" and Regulation (EC) No 641/2004 of 6 April 2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003".

² The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995); and ISO 5725 (1994).

Quality assurance

The EU-RL GMFF is ISO 9001:2008 certified (certificate number: CH-32232) and ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EU-RL GMFF quality system.

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1. Timeline

In line with Regulation (EC) No 1829/2003, Dow AgroSciences LLC provided the EU-RL GMFF with a copy of the official application for authorisation of an event-specific method for the detection and quantification of maize event DAS-40278-9 (unique identifier DAS-40278-9) together with genomic DNA as negative and positive control samples (October 2010).

In response to an early submission of the method, the EU-RL GMFF started its step-wise validation procedure (step 1: dossier reception) already in advance to the official dossier (December 2010), before EFSA declared the dossier as complete and valid (June 2011).

The scientific dossier assessment (step 2) focused on the reported method performance characteristics assessed against the ENGL method acceptance criteria³ (see http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf for a summary of method acceptance criteria and method performance requirements). It was positively concluded in February 2011.

In step 3 of the validation procedure (Experimental testing), the EU-RL GMFF verified the purity of the control samples and conducted an in-house testing of the method provided. The positive and negative control DNA samples - submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Regulation (EC) No 1829/2003 - were found extensively degraded. Further to a replacement request, new control samples were received and considered (July 2011). The method characteristics were verified in-house by quantifying five blind GM levels within the range of 0.1%-5% on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision were within the limits established by the ENGL.

In addition, and in line with the requirements of Reg. (EU) No 619/2011, the EU-RL GMFF also verified *i)* the zygosity ratio of the positive control sample submitted by investigating the GM- to reference- target ratio by means of digital PCR, in order to determine the conversion factor between copy numbers and mass fractions; and *ii)* the method's precision (relative repeatability standard deviation, RSDr %) at the 0.1% related to mass fraction of GM-material on fifteen replicates. Step 3 was finished in June 2012 with the conclusion to enter into a collaborative trial (step 4).

The collaborative trial (step 4) was organised and took place in October/November 2011. It demonstrated that the method is well suited for identifying the presence of GM event DAS-40278-9 in DNA samples and is therefore applicable for this purpose.

³ EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>)

2. Step 1 (dossier acceptance) and step 2 (scientific dossier assessment)

The early submitted dossier was found to be complete (step 1) and the verification against the formal dossier did not reveal any difference.

In step 2, documentation and data provided by the applicant were evaluated by the EU-RL GMFF for compliance with the ENGL method acceptance criteria. The parameters of the calibration curves (slope, R^2 coefficient) were determined by the applicant by quantifying in eight runs five test samples at different GM-levels expressed in haploid genome copy number (see Table 1).

Table 1. Values of slope and R^2 obtained by the applicant

	DAS-40278-9		<i>hmg</i>	
	Slope	R^2	Slope	R^2
Run 1	-3.30	1.00	-3.42	1.00
Run 2	-3.37	1.00	-3.33	1.00
Run 3	-3.23	1.00	-3.42	1.00
Run 4	-3.21	1.00	-3.45	1.00
Run 5	-3.20	1.00	-3.39	1.00
Run 6	-3.20	1.00	-3.41	1.00
Run 7	-3.40	1.00	-3.43	1.00
Run 8	-3.30	1.00	-3.38	1.00

Table 1 indicates that the slope and R^2 coefficient of the standard curves for the GM-system (DAS-40278-9) and the maize-specific reference system (*hmg*) were within the ENGL acceptance criteria (slope between -3.1 and -3.6 and R^2 coefficient ≥ 0.98).

Table 2. Mean %, precision and trueness (measured at five GM-levels by the applicant)

Expected GMO %	Test results				
	0.08	0.5	0.9	2.0	5.0
Measured mean %	0.069	0.48	0.87	2.1	5.1
Precision (RSDr %)	15	11	9.4	9.1	9.6
Trueness (bias %)	-14	-4.0	-3.3	5.0	2.0

Table 2 reports precision and trueness for the five GM-levels tested by the applicant. Sixteen values for each GM-level were provided. Both parameters were within the ENGL acceptance criteria (trueness $\pm 25\%$, RSDr $\leq 25\%$ across the entire dynamic range).

3. Materials and methods

3.1 DNA extraction

Genomic DNA was isolated by the applicant from ground maize seeds and grains using a "CTAB-Wizard" protocol previously submitted for detection of event TC1507. This method had already been evaluated and tested by the EU-RL GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on testing were published in 2007 at <http://gmo-crl.jrc.ec.europa.eu/summaries/TC1507-DNAextrc.pdf>. In agreement with the ENGL position, which endorses the modularity principle (see also Annex I to Reg. (EC) No 641/2004), and given the similarity in the matrix, the EU-RL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for maize event DAS-40278-9.

3.2 Method protocol for the PCR analysis

The PCR method provided by the applicant and subsequently validated by the EU-RL GMFF (see <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>) is an event-specific, quantitative, real-time TaqMan[®] PCR procedure for the determination of the relative content of GM event DAS-40278-9 DNA to total maize DNA. The procedure is a simplex system, in which a maize *hmg* (*high mobility group*) specific assay, and the target assay (DAS-40278-9) are performed in separate wells.

For the detection of GM event DAS-40278-9, a 98-bp fragment of the region spanning the 5' plant-to-insert junction in maize DAS-40278-9 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and TAMRA (6-carboxytetramethylrhodamine) as quencher dye at its 3' end.

For the relative quantification of GM event DAS-40278-9, a maize specific reference system amplifies a 79-bp fragment of *high mobility group* (*hmg*), a maize endogenous gene, using *hmg* gene-specific primers and an *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end, and TAMRA as quencher at its 3' end.

Standard curves are generated for both the DAS-40278-9 and the *hmg* systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For relative quantification of event DAS-40278-9 DNA in a test sample, the DAS-40278-9 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 to obtain the percentage value ($GM\% = DAS-40278-9/hmg \times 100$).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (nanograms) by the published average 1C value for the maize genome (2.725 pg) (Arumuganathan & Earle, 1991). The copy number values used in the quantification, the GMO contents of the calibration samples and total DNA quantity used in PCR are listed in Table 3.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4
Total amount of DNA in reaction (ng)	200	40	8	1.33
Target taxon <i>hmg</i> copies	73394	14679	2936	489
DAS-40278-9 Maize GM copies	7339	1468	294	49

3.3 EU-RL GMFF experimental testing (step 3)

3.3.1 Determination of the zygosity ratio in the positive control sample

Annex II of Reg. (EU) No 619/2011 requires that “when results are primarily expressed as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EU-RL GMFF.” In order to satisfy this requirement, the EU-RL GMFF conducted an assessment of the zygosity (GM-target to reference target ratio) in the positive control sample submitted by the applicant.

To this end, the copy number of the DAS-40278-9 and of the *hmg* targets in the positive control sample were determined by digital PCR (dPCR), performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Genomic DNA was provided by the applicant as positive control sample for DAS-40278-9 maize event.

Two micrograms of genomic DNA were digested at 37 °C overnight with 20 units of the six-base cutter restriction enzyme Hind III. The latter does not cleave within the annealing sites for the primers of the DAS-40278-9 or *hmg* amplification systems. Hind III restriction sites are located outside the respective targeted sequences. Further to digestion, the DNA was precipitated with ammonium acetate 2.5 M and two volumes of absolute ethanol. The outcome of enzymatic digestion was controlled by running approximately 200 nanograms of Hind III digested and 200 nanograms of undigested DNA in comparison with a DNA molecular marker in 1% agarose-gel electrophoresis. Digested template DNA was used in digital PCR experiments. Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix

with (no UNG, Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe, at the reaction concentrations indicated in the corresponding Validated Method (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>), 1 µL of DNA at a concentration of 0.5 ng/µL, suitable to avoid panel saturation after analysis (optimal between 200<positive partitions<500).

Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). A volume of 9 µL of reaction mix was loaded into each well of which only approximately 4.6 µL were distributed into the 765 chambers (or partitions) constituting one panel. The analysis was repeated three times; five replicates in five panels were run each time for both the GM- and reference-assay, with a total number of fifteen data sets for both targets. No template controls were included. Amplification conditions were as reported in the Validated Method (see Annex). Data analysis and copy number calculation was performed using the BioMark digital PCR Analysis software, the range of Ct retention was from 20 to 40.

Calculations of means and variances were carried out according to the procedure outlined for random variables in Annex 4 of the ENGL guidance document 'Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods'⁴.

3.3.2 In-house verification of the method performance against ENGL method acceptance criteria

The method performance characteristics were verified (EU-RL GMFF step 3) by quantifying on a copy number basis five blinded test samples containing a range of 0.1%-5% GM levels. The experiments were performed on an ABI 7900 real-time platform under repeatability conditions and followed the protocol described in the material and method section. Test samples with GM-levels 5.0%, 2.0%, 0.9% and 0.4% were tested in two real-time PCR runs with two replicates for each GM-level on each plate (total of four replicates per GM-level). The test sample with GM-level 0.1% was tested in 15 replicates in an additional run. Average values of the slope and of the R² coefficient of the standard curves and method trueness and precision over the dynamic range were evaluated against the ENGL method acceptance criteria.

In order to assess the method compliance with Reg. (EU) No 619/2011, the EU-RL GMFF determined the zygosity of the GM-insert in the positive control sample and estimated, based on 15 replicates, the method precision (RSDr) at 0.1% GM level in mass fraction.

3.4 International collaborative study (step 4)

The international collaborative study (EU-RL GMFF step 4) involved twelve laboratories, all being "National reference laboratories, assisting the CRL for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 1981/2006. The study was carried out in accordance with the following internationally accepted guidelines:

⁴ Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. European Network of GMO laboratories (ENGL), 2011.
<http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf>

1. The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995)
2. ISO 5725 (1994)

The objective of the international collaborative study was to verify in experienced laboratories the trueness and precision of the PCR analytical method that was provided by the applicant and which is described under 3.2, above and in the attached "Validated Method".

3.4.1 List of participating laboratories

The participants in the DAS-40278-9 validation study were randomly selected from the 31 national reference laboratories (NRL) that offered to participate.

Clear guidance was given to the selected laboratories to strictly follow the standard operational procedures that were provided for the execution of the protocol (the protocol is detailed in the Validated Method, available in Annex and at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>). The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the validation of the detection method for maize DAS-40278-9

Laboratory	Country
Agricultural Institute of Slovenia	SI
BioGEVES	FR
Central Agricultural Office	HU
Federal Institute for Risk Assessment	DE
Institute for Agricultural and Fisheries Research	BE
Food, Veterinary and Environmental Diagnostic Institute	DE
National Institute of Biology	SI
National Research Institute of Animal Production,	PL
National Veterinary Research Institute	PL
Plant Health Laboratory	FR
Service Commun des Laboratoires du MINEFI	FR
Walloon Agricultural Research Centre	BE

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used a range of real-time PCR equipment: seven laboratories used the ABI 7500, three used the ABI 7900, one used the Roche LC480 and one used the Stratagene Mx 3005P.

The variability of equipment, with its known potential influence on PCR results, reflects the real situation in the control laboratories and provides additional assurance that the method is robust and useable under real conditions.

3.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method, control samples were provided by the EU-RL GMFF to the participating laboratories. They were derived from:

- i) genomic DNA extracted by the applicant from homozygous maize seeds harbouring the event DAS-40278-9, and
- ii) genomic DNA extracted by the applicant from conventional maize seeds genetically similar to those harbouring the DAS-40278-9 event.

The control samples were prepared by the EU-RL GMFF from the genomic DNA provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11⁵.

These positive and negative control samples were also used by the EU-RL GMFF to prepare standard (of known GMO-content) and test samples (of unknown GM-content), containing mixtures of DAS-40278-9 maize DNA and non-GM maize DNA, as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes.

The calibration sample S1 was prepared by mixing the appropriate amount of DAS-40278-9 DNA with control non-GM maize DNA to obtain a 10% GM solution. Calibration samples S2-S3 were prepared by 5-fold dilution from the S1 sample and the S4 sample was prepared by 6-fold dilution from the S3 sample.

The 12 NRLs participating in the validation study received the following materials:

- ✓ Four calibration samples with known concentrations of GM-event (175 µL of DNA solution each) labelled from S1 to S4 (Table 3).
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution each at 35ng/µL) labelled from U1 to U20, representing 5 GM levels (Table 5).

⁵ Control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample). Reg. (EC) No 1829/2003, Art. 2 (11).

Table 5. DAS-40278-9 GM contents in genome copy number

DAS-40278-9 GM%
GM copy number/maize genome copy number x 100
5.00
2.00
0.90
0.40
0.10

✓ Reaction reagents:

- TaqMan® Universal PCR Master Mix no UNG (2x), one vial: 8 mL
- distilled sterile water, one vial: 3.5 mL

✓ Primers and probes (1 tube each) as follows:

hmg taxon-specific assay

- MaiJ-F (10 µM): 280 µL
- mhmg-R (10 µM): 280 µL
- mhmg-P (10 µM): 144 µL
-

DAS-40278-9 assay

- DAS-40278-9_5'-f1 (10 µM): 280 µL
- DAS-40278-9_5'-r3 (10 µM): 280 µL
- DAS-40278-9_5'-S2 (10 µM): 120 µL

3.4.4 Design of the collaborative study

Participant laboratories received a detailed validation protocol, that included, inter alia, the exact design of the PCR plates, ensuring that on each PCR plate the samples were analysed for the DAS-40278-9 specific system and for the *hmg* taxon-specific system. In total, two plates were run per each participating laboratory.

The laboratories prepared the master-mixes for the DAS-40278-9 and *hmg* assays in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per determined plate lay-out. The amplification reactions followed the cycling program specified in the protocol and the raw data were reported to the EU-RL on an excel sheet that was designed, validated and distributed by the EU-RL GMFF. Participants determined the GM% in the test samples according to the instructions and using the excel sheet provided. All data are stored by the EU-RL GMFF on a dedicated and protected server.

The EU-RL GMFF analysed the data against the parameters and the limits set by the ENGL, i.e. trueness, precision, amplification efficiency and linearity.

3.4.5 Deviations reported from the protocol

Eleven laboratories reported no deviations from the method protocol.

One laboratory performed the PCR reactions in a total volume of 20 µL because a 384-well plate configuration of the ABI 7900HT instrument was used. However, the final concentrations of the PCR reagents and the volume of DNA loaded per reaction remained unchanged.

4. Results

4.1 EU-RL GMFF experimental testing

4.1.1 Determination of the zygosity ratio in the positive control sample

The results of the tests to determine the zygosity ratio in the positive control samples are shown in Table 6.

Table 6. Summary of dPCR analysis conducted on the DAS-40278-9 and *hmg* targets in the positive control sample.

Mean ratio (DAS-40278-9/<i>hmg</i>)	0.991
Standard deviation	0.067
RSD_r (%)	6.8
Standard error of the mean	0.017
Upper 95% CI of the mean	1.028
Lower 95% CI of the mean	0.954

In conclusions, the 95% confidence interval (CI) spans around 1 and therefore the mean ratio is not significantly different from an expected ratio of 1, assuming a GM homozygous and a single-copy reference target, for an alpha = 0.05.

Hence:

$$\text{GM \% in DNA copy number ratio} = \text{GM \% in mass fraction}$$

4.1.2 In-house verification of method performance against ENGL method acceptance criteria

Test samples with GM-levels 5.0%, 2.0%, 0.9% and 0.4% were tested by the EU-RL GMFF in two real-time PCR runs (run A and B) with two replicates for each GM-level on each plate (total of four replicates per GM-level). The sample with a 0.1% GM-level was tested in 15 replicates in one run (run C). The corresponding standard curve parameters are shown in Table 7 and 8.

Table 7. Standard curve parameters

	DAS-40278-9 method			<i>hmg</i> reference method		
	Slope	PCR efficiency*	R ²	Slope	PCR efficiency*	R ²
Run A	-3.36	98	1.00	-3.35	99	1.00
Run B	-3.28	102	1.00	-3.36	98	1.00
Run C	-3.31	101	0.99	-3.30	101	1.00

* PCR efficiency (%) is calculated using the formula: Efficiency = $(10^{(-1/\text{slope})} - 1) \times 100$

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R² coefficient shall be ≥ 0.98 .

Table 7 documents that the slopes of the standard curves, and the R² coefficients were within the limits established by the ENGL.

Table 8. Outcome of the in-house tests, with regards to the quantification of the five test samples

Target GM-level %	Measured GM %	Bias %	Precision (RSDr %)
5.0	5.17	3.44	5
2.0	1.93	-3.40	0
0.9	0.87	-3.24	11
0.4	0.40	-0.79	7
0.1	0.11	11.65	19

According to the ENGL method acceptance criteria the method's trueness, measured as bias %, should be within $\pm 25\%$ of the target value over the entire dynamic range. The method's precision, estimated as RSDr % (relative standard deviation of repeatability) should be $\leq 25\%$ over the dynamic range. Table 8 documents that trueness and precision of quantification were within the limits established by the ENGL.

4.2 Results of the international collaborative study

4.2.1 PCR efficiency and linearity

Standard curve slopes (from which the PCR efficiency (%) is calculated using the formula: Efficiency = $(10^{(-1/\text{slope})} - 1) \times 100$), and R² values (expressing the linearity of the regression)

reported by participating laboratories for the DAS-40278-9 and the *hmg* assays are provided in Table 9.

Table 9. Values of slope, PCR efficiency and R^2 obtained during the validation study

Lab	Plate	DAS-40278-9			<i>hmg</i>		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.29	101.48	1.00	-3.40	96.84	1.00
	B	-3.33	99.50	1.00	-3.37	97.91	1.00
2	A	-3.40	96.82	1.00	-3.36	98.27	1.00
	B	-3.35	98.68	1.00	-3.37	97.90	1.00
3	A	-3.44	95.29	1.00	-3.44	95.34	1.00
	B	-3.36	98.30	1.00	-3.40	96.70	1.00
4	A	-3.48	93.81	1.00	-3.45	94.82	1.00
	B	-3.43	95.50	0.99	-3.52	92.42	1.00
5	A	-3.43	95.56	1.00	-3.43	95.70	1.00
	B	-3.49	93.39	1.00	-3.42	95.92	1.00
6	A	-3.57	90.46	1.00	-3.66	87.47	1.00
	B	-3.56	90.90	1.00	-3.49	93.35	1.00
7	A	-3.08	111.29	1.00	-3.41	96.29	1.00
	B	-3.17	106.91	1.00	-3.39	97.40	1.00
8	A	-3.03	113.95	0.99	-3.32	100.02	1.00
	B	-2.94	118.76	0.99	-3.19	105.60	0.99
9	A	-3.29	101.22	1.00	-3.66	87.69	1.00
	B	-3.46	94.47	0.99	-3.44	95.42	1.00
10	A	-3.05	112.71	0.99	-3.55	91.13	0.99
	B	-3.05	112.77	1.00	-3.37	97.94	1.00
11	A	-3.46	94.44	1.00	-3.57	90.56	1.00
	B	-3.23	104.02	0.99	-3.60	89.57	1.00
12	A	-3.61	89.32	1.00	-3.50	93.05	1.00
	B	-3.36	98.45	1.00	-3.51	92.88	1.00
Mean		-3.33	100.33	1.00	-3.45	95.01	1.00

Table 9 indicates that the efficiency of amplification for the DAS-40278-9 system ranges from 89 to 119 and the linearity from 0.99 to 1.00; the amplification efficiency for the maize-specific system ranges from 87% to 106% and the linearity is again about 1.00. The mean PCR efficiency was 100% for the DAS-40278-9 assay and 95% for the *hmg* assay. Both values were within the ENGL acceptance criteria. The average R^2 of the methods was 1.00 for both the DAS-40278-9 and *hmg* assays.

These results confirm the appropriate performance of the methods tested in terms of efficiency and linearity.

4.2.2 GMO quantification

Table 10 reports the values of the four replicates for each GM level as provided by all laboratories.

Table 10. GM% values determined by laboratories for test samples

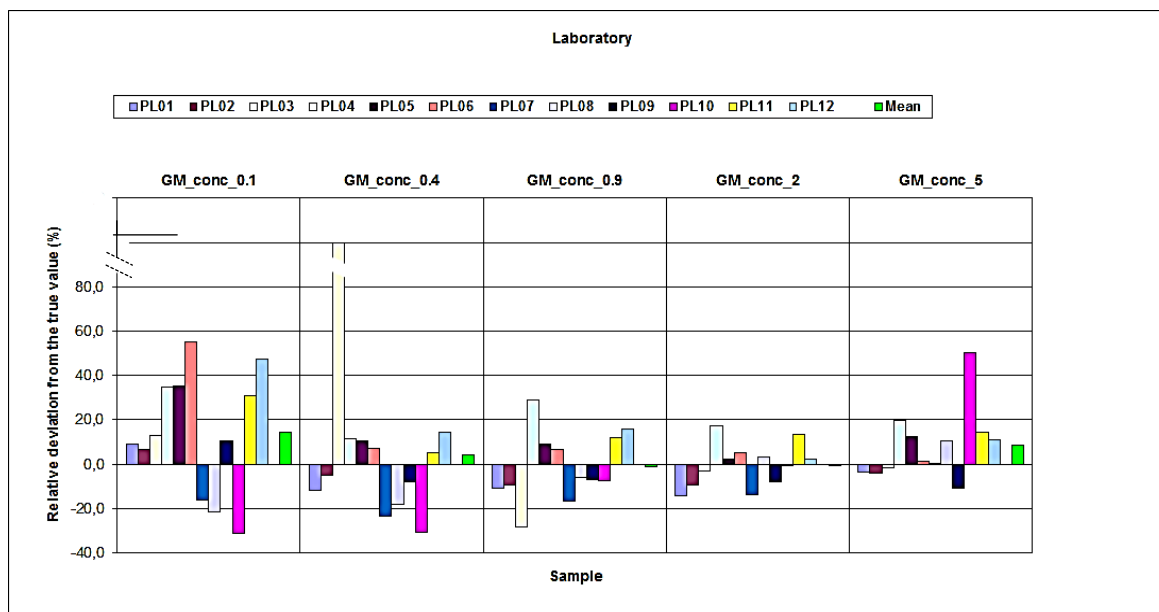
	GMO content GMO% = GMO copy number/maize genome copy number x 100 and GM DNA mass/maize DNA mass x 100																			
LAB	0.1				0.4				0.9				2.0				5.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.10	0.11	0.12	0.11	0.41	0.33	0.33	0.33	0.71	0.75	0.97	0.78	1.87	1.66	1.70	1.62	4.51	4.81	4.99	4.93
2	0.11	0.10	0.09	0.12	0.39	0.38	0.39	0.37	0.80	0.77	0.85	0.84	1.86	1.87	1.69	1.82	4.59	5.17	4.48	4.98
3	0.10	0.12	0.11	0.13	0.36	0.38	5.77	0.37	0.83	0.00	0.78	0.96	2.03	1.94	1.84	1.94	4.95	4.89	5.20	4.69
4	0.12	0.12	0.15	0.15	0.41	0.49	0.46	0.43	1.30	1.01	1.19	1.14	2.32	2.25	2.50	2.32	6.24	5.51	5.32	6.82
5	0.13	0.15	0.14	0.13	0.46	0.44	0.43	0.44	0.98	1.01	0.96	0.96	2.05	2.08	2.08	1.99	6.06	5.05	5.73	5.66
6	0.15	0.18	0.16	0.13	0.46	0.35	0.44	0.46	0.91	0.92	0.99	1.02	2.68	2.01	1.78	1.96	4.89	4.09	5.48	5.83
7	0.08	0.11	0.08	0.07	0.32	0.30	0.30	0.30	0.74	0.70	0.75	0.82	1.73	1.76	1.65	1.75	4.97	4.86	5.10	5.10
8	0.07	0.07	0.08	0.08	0.24	0.34	0.37	0.35	0.76	0.87	0.78	0.98	2.09	2.12	2.00	2.03	5.27	5.62	5.82	5.44
9	0.09	0.09	0.13	0.14	0.29	0.35	0.45	0.38	0.68	0.68	1.02	0.96	1.70	1.49	2.23	1.95	5.11	3.72	3.64	5.38
10	0.07	0.07	0.06	0.07	0.25	0.32	0.26	0.28	0.85	0.90	0.92	0.66	1.78	2.42	2.03	1.73	7.30	0.00	5.56	9.69
11	0.14	0.16	0.12	0.11	0.47	0.46	0.38	0.37	1.04	1.07	0.93	0.97	2.78	2.29	2.10	1.92	3.71	7.27	7.24	4.68
12	0.11	0.14	0.17	0.17	0.45	0.43	0.47	0.47	0.95	0.98	1.12	1.12	2.08	1.80	2.19	2.11	5.42	5.37	5.60	5.80

Data of all replicates were retained for the statistical analysis and for tests of outliers (Cochrane and Grubbs) whose results are reported in Table 11.

A graphical representation of the data reported in Table 10 is provided in Figure 1.

In figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the deviation of the GM level measured by the respective laboratory in % of the true GM level; the green bar on the right represents the mean relative deviation over all twelve participating laboratories for each true GM level.

Figure 1. Relative deviation (%) from the true value of DAS-40278-9 for all laboratories*



*PL10 at GM level 2.0%, PL 7 at GM level 5.0% had very small relative deviations from the true value and the corresponding histograms do not show up in Figure 1. PL: participating laboratory.

Overall a trend can be observed to overestimate the GM-content at the lowest level. One laboratory overestimated the GM-content of sample 0.4% by almost 330%; this was due to one of the four replicates quantifying extremely high levels compared to the other three replicates which on the contrary, showed acceptable quantifications.

The mean bias generated by all laboratories is slightly over-predictive (+15%) at the 0.1% GM-level, and within $\pm 5\%$ at all other GM-levels. The method is therefore well within the accepted limits established by the ENGL.

All data were retained to feed the statistical analysis reported in Table 11.

5. Method performance requirements

Among the method performance requirements established by ENGL and adopted by the EU-RL GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>), repeatability and reproducibility are to be assessed through an international collaborative trial. Table 11 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the study.

According to the ENGL method performance requirements, the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35% over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 11, the method satisfies this requirement at all GM levels tested. Indeed, the highest value of RSD_R is 27% at the 0.1% GM level, thus within the acceptance criterion.

Table 11. Summary of validation results for the DAS-40278-9 detection and quantification method, expressed as GM-DNA copy numbers in relation to target taxon specific-DNA copy numbers

	Test Sample Expected GMO %				
	0.1	0.4	0.9	2.0	5.0
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	1	1	0	2
Reason for exclusion	-	C	C	-	2 C
Mean value of measured GM-content	0.12	0.38	0.91	2.0	5.2
Relative repeatability standard deviation, RSD_r (%)	15	11	11	11	9
Repeatability standard deviation	0.02	0.04	0.10	0.22	0.49
Relative reproducibility standard deviation, RSD_R (%)	27	19	17	14	12
Reproducibility standard deviation	0.03	0.07	0.15	0.27	0.63
Bias (absolute value)	0.015	-0.018	0.012	-0.009	0.177
Bias (%)	15	-4.4	1.3	-0.5	3.5

C= Cochran's test; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2. Bias is estimated according to ISO 5725 data analysis protocol.

Table 11 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for a collaborative study, the EU-RL GMFF requires that the RSD_r value is below 25%, as indicated by the ENGL (see Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>). As it can be observed from the values reported, the repeatability standard deviation is below 25% at all GM levels, with the highest value of 15% at the 0.1% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be $\pm 25\%$ across the entire dynamic range. In this case, the method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 15% at the 0.1% GM level.

6. Compliance of the method of detection of event DAS-40278-9 with the requirements of Regulation (EU) No 619/2011

To verify the compliance of the method under validation with the requirements of Regulation (EU) No 619/2011, the following steps were carried out and their outcome is summarised in Table 12:

- at step 2 of the validation process (scientific assessment of the dossier), the EU-RL GMFF concluded that it could accept the applicant's data on method performance and therefore accepted that the RSDr at the level of 0.08% in terms of GM-DNA copy numbers in relation to target taxon specific DNA copy numbers resulted to be 15% on 16 replicates (Table 2), hence below 25%;
- at step 3 of the validation process (in-house testing of the method), the EU-RL GMFF determined the RSDr at the level of 0.1% related to mass fraction of GM-material on the basis of fifteen replicates carried out under repeatability conditions. The RSDr was 19% (Table 8), hence below 25%;
- further to the conclusion of step 4 of the validation process (ring trial), the EU-RL GMFF analysed the data generated by the 12 participating laboratories for determining the method performance parameters. It found that the RSDr of the method at the level of 0.1% related to mass fraction of GM-material was 15%, therefore below the limit of 25%.

Table 12. Precision of the event-specific method for quantitative detection of DAS-40278-9 at or around 0.1% level related to mass fractions of GM material

Source	RSDr %	GM %
Applicant's method optimisation*	15 %	0.08 %
EU-RL GMFF tests	19 %	0.1 %
Collaborative study	15 %	0.1 %

* GM- DNA copy numbers in relation to target taxon specific DNA copy numbers

Based on the results of the EU-RL GMFF in-house verification and of the collaborative study, it is concluded that the method RSDr is equal or less than 25% at the level of 0.1% related to mass fraction of GM-material, hence the method meets the requirement laid down in Regulation (EU) No 619/2011.

7. Conclusion

The method provided by the applicant and described in detail under 3.2 (the Validated method is also available in Annex 1 and at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>) has been validated in accordance to the EU-RL GMFF validation scheme, respecting all requirements of the relevant EU legislation and international standards.

The dossier was found complete (step 1) and the scientific dossier analysis (step 2) concluded that the method appeared to meet the ENGL minimum performance criteria for a quantitative PCR method for detection and quantification of GM events for entering into validation.

The subsequent in-house verification of the method (step 3) by the EU-RL GMFF confirmed this conclusion.

The results of the international collaborative study (step 4) also indicated that the method meets all acceptance criteria and method performance requirements recommended by the ENGL (as detailed at <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>) for a valid quantitative PCR method for detection and quantification of GM-events.

In conclusion, the validation study confirmed that the method is applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004 and (EU) No 619/2011. The EU-RL GMFF further concludes that the method, if carried out in accordance with the validated method protocol (see Annex 1 and at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>) is applicable to appropriately extracted maize DNA.

8. References

1. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method - performance studies, *Pure & Appl. Chem.* 67, 331-343.
2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
3. Arumuganathan K. and Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9, 208-218.

Annex 1: Event-specific Method for the Quantification of Maize DAS-40278-9 by Real- time PCR

Validated Method

**Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit**

Method development:

Dow AgroSciences LLC

Method validation:

European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF)

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1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR (polymerase chain reaction) procedure for the determination of the relative content of maize event DAS-40278-9 DNA to total maize DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of maize event DAS-40278-9, a 98-bp fragment of the region spanning the 5' insert-to-plant junction in maize DAS-40278-9 event is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as a reporter at its 5' end, and the dye TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3' end.

For the relative quantification of maize event DAS-40278-9 DNA, a maize-specific reference system amplifies a 79-bp fragment of *high mobility group (hmg)*, a maize endogenous gene (Accession number, GeneBank: AJ131373.1), using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end, and TAMRA as quencher at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of DAS-40278-9 DNA in a test sample, Ct values for the DAS-40278-9 and *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DAS-40278-9 DNA to total maize DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional maize seeds. Precision and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF).

A detailed validation report can be found at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.04% in 200 ng of total maize DNA. The relative LOD was not assessed in the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.08% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1%.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' insert-to-plant junction in maize DAS-40278-9; the sequence is specific to event DAS-40278-9 and thus imparts event-specificity to the method.

The specificity of the maize taxon-specific assay was assessed by the method developer in real-time PCR using 100 ng of conventional genomic DNA extracted from maize, soybean, rapeseed, cotton, wheat, rice, potato, tomato, sugar beet, oat, barley, spelt, rye and sorghum. According to the method developer the maize-specific reference system did not react with any target DNA except the positive control.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA (100 ng) extracted from DAS-40278-9 (1%) as positive control sample and from maize DAS-40474-7, GA21, Bt176, Bt11, NK603, MON863, TC1507, MIR604, 59122, T25, MON810, MON88017, 3272, MON89034; sugar beet H7-1; rapeseed Ms8, Rf3, T45, RT73; cotton MON531, MON15985, MON1445, GHB614, LLcotton 25; potato EH-92-527-1; soybean DAS-64209-9, DAS-68416-4, A2704-12, DP-305423-1, DP-356043-5, GTS 40-3-2, MON89788, A5547-127; rice LL62; conventional maize, soybean, rapeseed, rice, cotton, wheat, potato and sugar beet.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan® probe of the DAS-40278-9 event showed no amplification signals following quantitative PCR analysis (45 cycles).

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- PCR reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps, unless specified otherwise, should be carried out at 0 – 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of maize event DAS-40278-9

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*hmg*) and for the GMO (event DAS-40278-9) target sequence is to be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated and is therefore not recommended.

The method is developed and validated for a total volume of 25 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curve has to be established on at least four samples. The first point of the calibration curve should be established for a sample containing 10% maize DAS-40278-9 DNA in a total of 200 ng of maize DNA (corresponding to approximately 73394 maize genome copies with one genome assumed to correspond to 2.725 pg of haploid maize genomic DNA)⁽¹⁾. Standards S2 to S3 are to be prepared by serial 5-fold dilution of the 10% standard. Standard S4 is to be prepared as a 6-fold dilution of the standard S3.

The copy number values of the calibration samples and total DNA quantity used in PCR are reported in Table 1.

Table 1. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4
Total amount of DNA in reaction (ng)	200	40	8	1.33
Target taxon <i>hmg</i> copies	73394	14679	2936	489
DAS-40278-9 Maize GM copies	7339	1468	294	49

A calibration curve is to be produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available within the sequence detection system software of the method user.

3.2.3 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.
2. To prepare the amplification reaction mixtures, add the following components (Table 2 and 3) in two reaction tubes (one for the DAS-40278-9 assay and one for the *hmg* assay) on ice and in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the DAS-40278-9 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix no UNG 2x	1x	12.5
DAS-40278-9_5'-f1 (10 µM)	350 nM	0.875
DAS-40278-9_5'-r3 (10 µM)	350 nM	0.875
DAS-40278-9_5'-S2 (10 µM)	150 nM	0.375
Nuclease free water	#	5.375
DNA	#	5
Total reaction volume:		25 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix no UNG 2X	1x	12.5
MaiJ-F (10 µM)	300 nM	0.75
mhmg-R (10 µM)	300 nM	0.75
mhmg-P (10 µM)	180 nM	0.45
Nuclease free water	#	5.55
DNA	#	5
Total reaction volume:		25 µL

- Mix well and centrifuge briefly.
- Prepare two reaction tubes (one for the maize DAS-40278-9 and one for the *hmg* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of reaction mix for 3.5 PCR repetitions (e.g. (70 µL for the *hmg* reference system and 70 µL for the DAS-40278-9 maize system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (e.g. 17.5 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.

6. Spin down the tubes in a micro-centrifuge. Aliquot 25 μ L in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4°C) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for DAS-40278-9/*hmg* methods.

Step	Stage	T (°C)	Time (s)	Acquisition	Cycles
1	Initial denaturation	95	600	No	1X
2	Amplification	Denaturation	95	No	45X
		Annealing & Extension	60	Yes	

3. 3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold: display the amplification curves of one assay (e.g. DAS-40278-9) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" button to ensure changes affect Ct values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.
- b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).
- c) Save the settings.
- d) Repeat the procedure described in a), b) and c) on the amplification plots of the other system (e.g. *hmg*).
- e) Save the settings and export all the data for further calculations.

3. 4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct-values for each reaction.

The standard curves are generated both for the *hmg* and the DAS-40278-9 specific assays by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the DNA copy number in the unknown sample.

To obtain the percentage value of event DAS-40278-9 DNA in the unknown sample, the DAS-40278-9 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 ($GM\% = DAS-40278-9/hmg \times 100$).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Centrifuge for PCR-plates
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan[®] Universal PCR Master Mix, No AmpErase[®] UNG. Applied Biosystems Part No 4326614.

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
DAS-40278-9			
Forward primer	DAS-40278-9_5'-f1	5' CAC gAA CCA TTg AgT TAC AAT C 3'	22
Reverse primer	DAS-40278-9_5'-r3	5' Tgg TTC ATT gTA TTC Tgg CTT Tg 3'	23
Probe	DAS-40278-9_5'-S2	5' 6FAM- CgT AgC TAA CCT TCA TTg TAT TCC g -TAMRA 3'	25
<i>hmg</i>			
Forward primer	MaiJ-F	5' TTg gAC TA g AAA TCT CgT gCT gA 3'	23
Reverse primer	mhmg-R	5' gCT ACA TA g ggA gCC TTg TCC T 3'	22
Probe	mhmg-P	5' 6FAM- CAA TCC ACA CAA ACg CAC gCg TA -TAMRA 3'	23

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine;

5. References

1. Arumuganathan K., Earle E.D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter*, 9: 208-218.



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit



Event-specific Method for the Quantification of Maize DAS-40278-9 using Real-time PCR

Validated Method

7 November 2012

Method development:

Dow AgroSciences LLC

Method validation:

European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF)

Quality assurance

The EU-RL GMFF is ISO 9001:2008 certified (certificate number: CH-32232) and ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EU-RL GMFF quality system.

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1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan[®] PCR (polymerase chain reaction) procedure for the determination of the relative content of maize event DAS-40278-9 DNA to total maize DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of maize event DAS-40278-9, a 98-bp fragment of the region spanning the 5' insert-to-plant junction in maize DAS-40278-9 event is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as a reporter at its 5' end, and the dye TAMRA (6-carboxytetramethylrhodamine) as a quencher dye at its 3' end.

For the relative quantification of maize event DAS-40278-9 DNA, a maize-specific reference system amplifies a 79-bp fragment of *high mobility group (hmg)*, a maize endogenous gene (Accession number, GeneBank: AJ131373.1), using *hmg* gene-specific primers and a *hmg* gene-specific probe labelled with FAM as reporter dye at its 5' end, and TAMRA as quencher at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of DAS-40278-9 DNA in a test sample, Ct values for the DAS-40278-9 and *hmg* systems are determined for the sample. Standard curves are then used to estimate the relative amount of DAS-40278-9 DNA to total maize DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional maize seeds. Precision and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF).

A detailed validation report can be found at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.04% in 200 ng of total maize DNA. The relative LOD was not assessed in the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.08% in 200 ng of total maize DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1%.

2.5 Molecular specificity

The method exploits a unique DNA sequence in the region spanning the 5' insert-to-plant junction in maize DAS-40278-9; the sequence is specific to event DAS-40278-9 and thus imparts event-specificity to the method.

The specificity of the maize taxon-specific assay was assessed by the method developer in real-time PCR using 100 ng of conventional genomic DNA extracted from maize, soybean, rapeseed, cotton, wheat, rice, potato, tomato, sugarbeet, oat, barley, spelt, rye and sorghum. According to the method developer the maize-specific reference system did not react with any target DNA except the positive control.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA (100 ng) extracted from DAS-40278-9 (1%) as positive control sample and from maize DAS-40474-7, GA21, Bt176, Bt11, NK603, MON863, TC1507, MIR604, 59122, T25, MON810, MON88017, 3272, MON89034; sugarbeet H7-1; rapeseed Ms8, Rf3, T45, RT73; cotton MON531, MON15985, MON1445, GHB614, LLcotton 25; potato EH-92-527-1; soybean DAS-64209-9, DAS-68416-4, A2704-12, DP-305423-1, DP-356043-5, GTS 40-3-2, MON89788, A5547-127; rice LL62; conventional maize, soybean, rapeseed, rice, cotton, wheat, potato and sugarbeet.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the TaqMan[®] probe of the DAS-40278-9 event showed no amplification signals following quantitative PCR analysis (45 cycles).

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.
- PCR reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps, unless specified otherwise, should be carried out at 0 – 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of maize event DAS-40278-9

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*hmg*) and for the GMO (event DAS-40278-9) target sequence is to be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated and is therefore not recommended.

The method is developed and validated for a total volume of 25 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curve has to be established on at least four samples. The first point of the calibration curve should be established for a sample containing 10% maize DAS-40278-9 DNA in a total of 200 ng of maize DNA (corresponding to approximately 73394 maize genome copies with one genome assumed to correspond to 2.725 pg of haploid maize genomic DNA)⁽¹⁾. Standards S2 to S3 are to be prepared by serial 5-fold dilution of the 10% standard. Standard S4 is to be prepared as a 6-fold dilution of the standard S3.

The copy number values of the calibration samples and total DNA quantity used in PCR are reported in Table 1.

Table 1. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4
Total amount of DNA in reaction (ng)	200	40	8	1.33
Target taxon <i>hmg</i> copies	73394	14679	2936	489
DAS-40278-9 Maize GM copies	7339	1468	294	49

A calibration curve is to be produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available within the sequence detection system software of the method user.

3.2.2 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.
2. To prepare the amplification reaction mixtures, add the following components (Table 2 and 3) in two reaction tubes (one for the DAS-40278-9 assay and one for the *hmg* assay) on ice and in the order mentioned below (except DNA).

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the DAS-40278-9 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix no UNG 2x	1x	12.5
DAS-40278-9_5'-f1 (10 µM)	350 nM	0.875
DAS-40278-9_5'-r3 (10 µM)	350 nM	0.875
DAS-40278-9_5'-S2 (10 µM)	150 nM	0.375
Nuclease free water	#	5.375
DNA	#	5
Total reaction volume:		25 µL

Table 3. Amplification reaction mixture in the final volume/concentration per reaction well for the maize *hmg* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix no UNG 2X	1x	12.5
MaiJ-F (10 µM)	300 nM	0.75
mhmg-R (10 µM)	300 nM	0.75
mhmg-P (10 µM)	180 nM	0.45
Nuclease free water	#	5.55
DNA	#	5
Total reaction volume:		25 µL

- Mix well and centrifuge briefly.
- Prepare two reaction tubes (one for the maize DAS-40278-9 and one for the *hmg* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of reaction mix for 3.5 PCR repetitions (e.g. 70 µL for the *hmg* reference system and 70 µL for the DAS-40278-9 maize system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (e.g. 17.5 µL DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
- Spin down the tubes in a micro-centrifuge. Aliquot 25 µL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4°C) to spin down the reaction mixture.

7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 4.

Table 4. Cycling program for DAS-40278-9/*hmg* methods.

Step	Stage	T (°C)	Time (s)	Acquisition	Cycles
1	Initial denaturation	95	600	No	1X
2	Amplification	Denaturation	95	No	45X
		Annealing & Extension	60	Yes	

3.3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold: display the amplification curves of one assay (e.g. DAS-40278-9) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" button to ensure changes affect Ct values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.
- b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).
- c) Save the settings.
- d) Repeat the procedure described in a), b) and c) on the amplification plots of the other system (e.g. *hmg*).
- e) Save the settings and export all the data for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct-values for each reaction.

The standard curves are generated both for the *hmg* and the DAS-40278-9 specific assays by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the DNA copy number in the unknown sample.

To obtain the percentage value of event DAS-40278-9 DNA in the unknown sample, the DAS-40278-9 copy number is divided by the copy number of the maize reference gene (*hmg*) and multiplied by 100 ($GM\% = \text{DAS-40278-9}/hmg \times 100$).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Centrifuge for PCR-plates
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan[®] Universal PCR Master Mix, No AmpErase[®] UNG. Applied Biosystems Part No 4326614.

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
DAS-40278-9			
Forward primer	DAS-40278-9_5'-f1	5' CAC gAA CCA TTg AgT TAC AAT C 3'	22
Reverse primer	DAS-40278-9_5'-r3	5' Tgg TTC ATT gTA TTC Tgg CTT Tg 3'	23
Probe	DAS-40278-9_5'-S2	5' 6FAM- CgT AgC TAA CCT TCA TTg TAT TCC g -TAMRA 3'	25
<i>hmg</i>			
Forward primer	MaiJ-F	5' TTg gAC TAG AAA TCT CgT gCT gA 3'	23
Reverse primer	mhmg-R	5' gCT ACA TAG ggA gCC TTg TCC T 3'	22
Probe	mhmg-P	5' 6FAM- CAA TCC ACA CAA ACg CAC gCg TA -TAMRA 3'	23

FAM: 6-carboxyfluorescein; TAMRA: carboxytetramethylrhodamine;

5. References

1. Arumuganathan K., Earle E.D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter*, 9: 208-218.

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Abstract

In line with its mandate the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific polymerase chain reaction (PCR) method for detecting and quantifying maize event DAS-40278-9 (unique identifier DAS-40278-9). The validation study was conducted according to the EU-RL GMFF validation procedure [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>] and the internationally accepted guidelines.

In accordance with current EU legislation¹, Dow AgroSciences LLC has provided the detection method and the positive and negative control samples (genomic DNA from maize seeds harbouring the DAS-40278-9 event as positive control DNA, genomic DNA from conventional maize seeds as negative control DNA). The EU-RL GMFF prepared the validation samples (calibration samples and blind samples at different GM percentage [DNA/DNA]), organised an international collaborative study and analysed the results.

The study confirms that the method meets the method performance requirements as established by the EU-RL GMFF and the ENGL in Annex I-2.C.2 to Regulation (EC) No 641/2004¹ and it fulfils the analytical requirements of Regulation (EU) No 619/2011.

This report is published at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>

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